EVALUATION OF NEUROPROTECTIVE ROLE OF GSK-3 β INHIBITORS IN CEREBRAL ISCHEMIC DAMAGE INDUCED BY MIDDLE CEREBRAL ARETREY OCCLUSION MODEL IN SPRAGUE DAWLEY RATS

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(Pharmacology)

Submitted By

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CERTIFICATE

This is to certify that the dissertation work entitled "'**EVALUATION OF NEUROPROTECIVE ROLE OF GSK-3β INHIBITORS IN CEREBRAL ISCHEMIC DAMAGE INDUCED BY MIDDLE CEREBRAL ARTERY OCCULUSION MODEL IN SPRAGUE DAWLEY RATS"** submitted by **University Reg. No.261525905** is a bonafide work carried out by the candidate under the guidance of **Dr. M. Ramanathan, M. Pharm., PhD.,** and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

Dr. M. Ramanathan, M. Pharm., PhD.,
Principal

DECLARATION

I do hereby declare that the dissertation work entitled "'**EVALUATION OF NEUROPROTECTIVE R0LE OF GSK-3β INHIBITORS IN CEREBRAL ISCHEMIC DAMAGE INDUCED BY MIDDLE CEREBRAL ARTERY OCCLUSION MODEL IN SPRAGUE DAWLEY RATS"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology**, was done by me under the guidance of **Dr. M. Ramanathan, M. Pharm., PhD.,** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017**.**

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled "'**EVALUATION OF NEUROPROTECIVE ROLE OF GSK-3β INHIBITORS IN CEREBRAL ISCHEMIC DAMAGE INDUCED BY SPRAGUE DAWLEY RATS"** submitted by **University Reg. No.261525905** is a bonafide work carried out by the candidate under the guidance of **Dr. M. Ramanathan, M. Pharm., PhD.,** and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

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Dedicated

To

Respectful Guide,

Beloved Parents

&

God

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Abbreviation

MCAO	**:**	Middle cerebral artery occlusion
GSK-3β	**:**	Glycogen synthase kinase -3β
CAMP	**:**	Cyclic adenosine monophosphate
MAO -B	**:**	Monoamine oxidase -B
BBB	**:**	Blood brain barrier
NADH	**:**	Nicotinamide adenine dinucleotide
TAK1	**:**	Transforming growth factor beta **-**activated kinase 1
PI3K	**:**	Phosphoinsotiyl 3 kinase
MMPS	**:**	Matrix metallo proteinases
nNOS	**:**	Neuron nitric oxide synthase
PAF	**:**	Platelet activating factor
AA	**:**	Arachidonic acid
NMDAR	**:**	N -methyl D-aspartate receptor
P53	**:**	Tumour suppressor protein
CBF	**:**	Cerebral blood flow
MCP-1	**:**	Monocyte chemoattractant protein -1
CREB	**:**	CAMP receptor element binding protein
ATP	**:**	Adenosine tri -phosphate
OGD	**:**	Oxygen and glucose deprivation

MPTP	**:**	(1 -Methyl -4-phenyl-1236-tetrahydropyridine
VGER –A	**:**	Vascular endothelial growth factor -A
PTP	**:**	Permeability transition pore
AD	**:**	Alzheimer disease
AMPK	**:**	Activated Mitogen protein kinase
ERK	**:**	Extracellular signal regulated kinase
MAPKS	**:**	Mitogen activated protein kinase
ROS	**:**	Reactive oxygen synthase
i NOS	**:**	Inducible nitric oxide synthase
TNF-α	**:**	Tumour necrosis factor – alpha
IAP	**:**	Inhibitors of apoptosis protein
e NOS	**:**	Endothelial nitric oxide synthase
n NOS	**:**	Neuronal nitric oxide synthase
ELISA	**:**	Enzyme linked immunosorbent assay
COX	**:**	Cyclooxygenase
NO	**:**	Nitrous oxide.
LO	**:**	Lipoxygenase
TRPVG	**:**	Transient receptor potential vanilloid -4
IL-1 β	**:**	Interleukin-1beta
AD	**:**	Alzheimer disease

AMPK	**:**	Activated Mitogen protein kinase
ERK	**:**	Extracellular signal regulated kinase
MAPKS	**:**	Mitogen activated protein kinase
ERK	**:**	Extracellular signal -regulated kinases

1.Introduction

1.INTRODUCTION

Glycogen synthase kinase 3 beta (GSK-3β) a serine threonine kinase has multiple functions, including regulating cellular development *(Luo et al., 2014:fan et al.,2014)* and tissue protection. GSK-3β is constitutively active in cells and its inactivation could be induced by phosphorylation at Ser-9 Growing evidence implicated that GSK-3β is involved in brain tissue protection against ischemic injury or brain trauma. A selective GSK-3β inhibitor could cause the phosphorylation at Ser-9 and inactivate GSK-3β, thus provided a protective effect against transient cerebral ischemic injury. The inhibition of GSK-3β was effective for regulating metabolic disorders. Moreover, acute GSK-3β inhibition was proved to be a novel therapeutic strategy for acute myocardial infarction in a diabetic model. Thus, GSK-3β signaling pathway might be involved in cerebral ischemic injury. Glycogen synthase kinase 3 (GSK-3β) dysregulation plays an important role in the pathogenesis of numerous disorders, affecting the central nervous system (CNS) encompassing both neuro inflammation *(Golpich et al.,2015)* and neurodegenerative diseases. Several lines of evidence have illustrated a key role of the GSK-3 and its cellular and molecular signaling cascades in the control of neuroinflammation. GSK-3β, one of the GSK-3 isomers, plays a major role in neuronal apoptosis and its inhibition decreases expression of alpha-Synuclei which make this kinase an attractive therapeutic target for neurodegenerative disorders.

Glycogen synthase kinase-3 (GSK-3), which is constitutively active and ubiquitously expressed in body tissues specially a *(Darshit et al.,2017)* brain neuron and glia.GSK-3β is expressed in most tissues. Expression of GSK-3β is abundant in major adult brain parts like cerebral cortex, striatum, hippocampus and cerebellum. GSK-3ß is found in all brain region with varying mRNA levels. GSK-3β is recognized as a crucial player in many cellular functions and its activity is tightly controlled by complex mechanisms that are dependent upon specific signalling pathways. Furthermore, GSK-3 gene dysfunction has been linked to a number of pathologies, including Alzheimer's disease (AD). In particular, the involvement of GSK-3β in several key pathophysiological pathways leading to AD and neurodegenerative diseases has placed this enzyme in a central position in the context of these disorders. GSK-3 appears to be a cellular nexus that integrates several signalling systems, including several second messengers and a wide selection of cellular stimulants.

GSK-3 acts as a regulator of apoptosis and inflammation, known contributors to stroke-induced cell death *(Venna et al.,2015)* Loss of GSK-3 β, not GSK-3alpha, suppressed

spontaneous neuronal death in extended culture models. GSK-3β inhibition reduces infarct size in adult stroke models and further interacted with pro-apoptotic transcription factors, such as p53, which upregulates cytochrome C release and Caspase-3 activity providing more evidence of GSK-3β's pro-apoptotic role following brain injury. Nonselective GSK-3 β inhibition with lithium is neuroprotective and GSK-3 β inhibitors are currently being tested in clinical trials for treatment of cognitive deficits and dementia. GSK-3beta is known to interact with the mitogen-activated protein kinase family (MAPKs) and promotes signalling after stress. Transforming growth factor-b-activated kinase-1 (TAK1) is a member of the MAPK family that is also known as mitogen-activated protein kinase kinase kinase-7. TAK1 is activated by TGF-b, tumour necrosis factor-alpha and other cytokines including interleukin-1 (IL-1). TAK is also as upstream kinase of 5′ adenosine monophosphate-activated protein kinase (AMPK), a key energy sensing kinase involved in stroke.

GSK-3 β dysregulation contributes to the pathogenesis of many disorders, such as several cancers and diabetes, *(Avrahami et al.,2013; Licht-Murava et al.,2013)* as well as neuroinflammatory and neurodegenerative diseases. Numerous studies have now implicated GSK-3 in control of various neuronal functions and have demonstrated that aberrant regulation of GSK-3 is involved in the etiology of neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Alzheimer's disease (AD), as well as in brain aging. . Abnormal regulation of GSK-3 activity is reported in patients with AD, ALS, major depression, schizophrenia and bipolar disorder.

Recent studies have suggested involvement of GSK-3β in cerebral ischemia. *(Darshit et al.,2017)* and demontsrated activation of GSK-3β (Tyr216 phosphorylation) was found directly correlated to cerebral ischemia. The report has shown GSK-3ß inhibition was beneficial in cerebral ischemic condition. GSK-3β inhibition has also shown neuroprotective effect by reducing infract size, Bax, caspase-3 and -9 activities both in *in vitro* and *in vivo* models of cerebral ischemia, suggesting a role of GSK-3β involved in the mitochondrial pathway in pathology. Research revealed the role of GSK-3β inhibition by NP00111, AR-A 014418, and lithium in hippocampal neuroprotection through ERK and PPAR-γ receptor signalling pathway in oxygen-glucose deprivation. This observation highlighted the link between GSK-3β, ERK, and PPAR- γ in cerebral ischemia. A study relating multiple pathways like calcium signalling (S100B - S100 calcium binding protein B) kinase pathway (MAPK/JNK-1, -2/p38), inflammatory pathway (NF-κβ, TNF-α, COX-2, iNOS and ICAM-

1), and typical mitochondrial apoptosis pathway (SOD, cytochrome c caspase-9 and Bcl-2) were shown to have direct relation with GSK-3β in cerebral ischemia. Inhibition of GSK-3β showed neuroprotection through down-regulation of these multiple pathways.

The objective of the present study is to evaluate the neuroprotective effect of GSK- 3β competitive and non competitive inhibitors in cerebral ischemic condition.

2. Literature review

STROKE

Stroke is a leading cause of death and disability, affecting 15 million people worldwide each year (***Zhang1 et al., 2015)***. Over 80% of stroke is caused by the obstruction of a cerebral artery by an endovascular embolus or thrombus formation. There are a number of underlying causes of stroke, including thrombosis, embolism,*(Tang et al.,2014: Vital et al.,2015)* stenosis and intracerebral hemorrhage. Embolic strokes are usually of extracranial origin and commonly result from clots formed in the heart due to atrial fibrillation. Thrombotic strokes are typically of intracranial origin and result from the rupture of an atheroma and subsequent injury of a downstream vessel, with the ensuing development of a thrombus in either a middle cerebral artery or a lacunar artery in the brain. Stroke recurrence occurs at a rate ranging between 4 and 14% and patients with risk factors, such as hypertension, are at higher risk of stroke recurrence. Up to 60–70% of recurrent strokes are of the same subtype as the initial stroke Ischemic stroke, due to interruption of the blood supply to the brain is one of the most important causes of morbidity and mortality worldwide. Currently, the control of systemic parameters, such as body temperature, blood pressure, and glycaemia, has considerably improved the outcome of stroke patients. In the absence of protective therapy, an early artery reperfusion, i.e. Mechanical or enzymatic thrombolysis, remains the primary goal of treatment for acute ischemic stroke.

CEREBRAL ISCHEMIA

Cerebral ischemia continues to be a major cause of death and the leading cause of long-term disability in humans. Acidotoxicity, ionic imbalance, and periinfarct depolarization also have a detrimental role in the initial phase of cerebral ischemia. Acidosis have long been recognized to aggravate brain injury associated with cerebral ischemia. A rapid drop of the pH to 6.5 or lower is frequently observed. The*(Darshitet al.,2017)*lack of oxygen supply promotes anaerobic glycolysis, which in turn leads to increased production of lactic acid. Accumulation of lactic acid, along with increased production of H+ from ATP hydrolysis, and release of H+ from presynaptic terminals, contribute to the acid build up in the brain and this leads to the activation of acid sensing ion channel (ASIC1a). Transport of sodium, calcium, and other ions is facilitated by pennexin 1 and 2 through P2X7 receptor, suggests that activation of both the receptors is found to be associated in cerebral ischemic stroke and aggravates acidosis and exitotoxicity. Both excitotoxicity and acidosis are linked to energy

crisis, oxidative stress, and apoptosis One of the crucial mediators of apoptosis involved in this cascade is GSK-3ß. Ischemia activated GSK-3ß by phosphorylation at Tyr216 up-regulates apoptotic markers like p53, bad, Bax, and destroys ß-catenine thereby arresting neurogenic transcription factor activation. Linking all sequences and exploring the interrelationship of ASIC1a, P2X7 receptor, and GSK-3ß might have a beneficial effect indifferent phases of ischemic stroke.

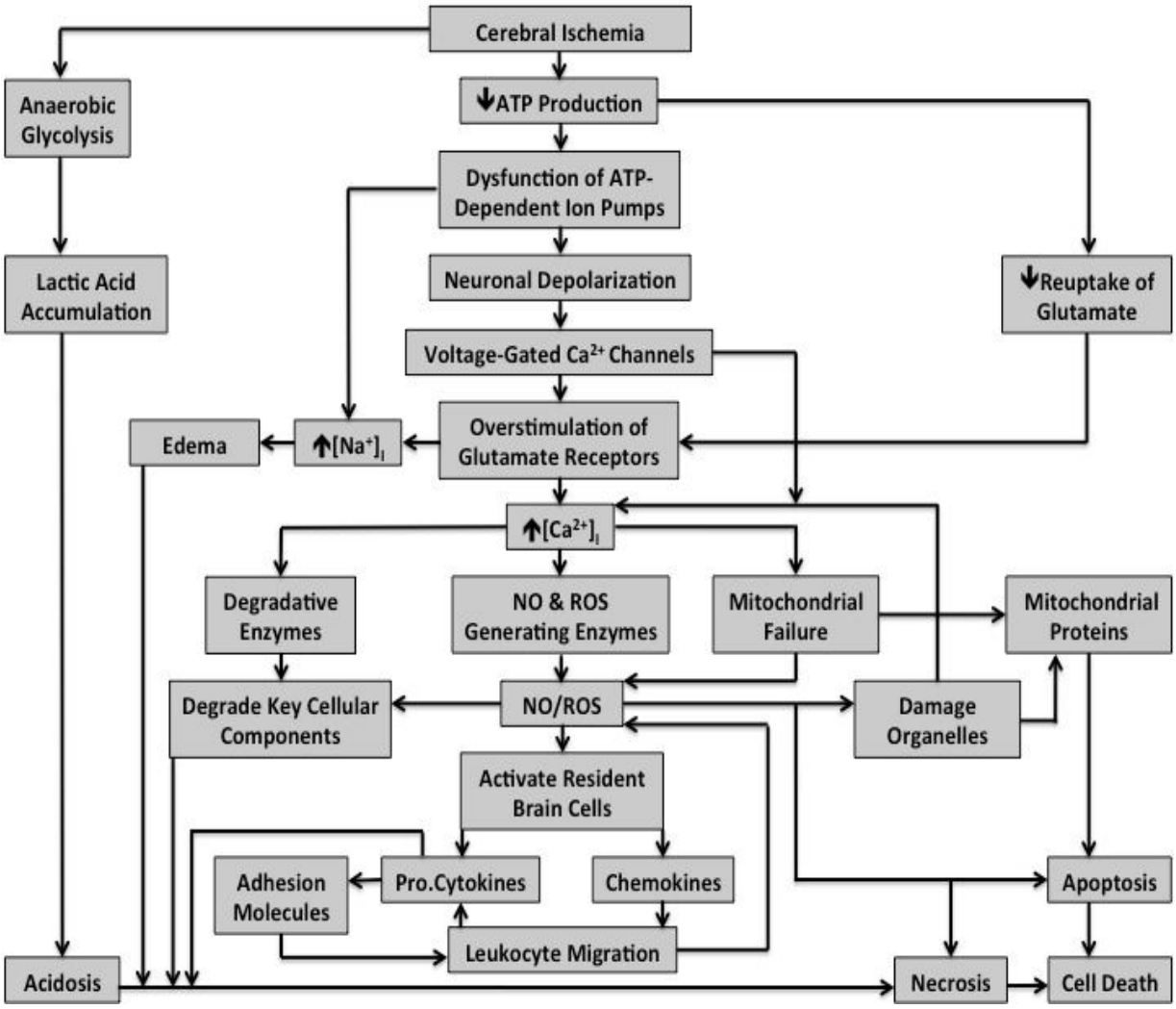


FIGURE.1. A schematic diagram of the major cell injury mechanisms involved in causing neuronal and glial cell death in cerebral ischemia. *(Mattson et al.,2000).*

TYPES OF CEREBRAL ISCHEMIA

1) Global cerebral ischemia (*Bacigaluppiet et al., 2010; Durukan&Tatlisuma et al., 2007).*

2) Focal cerebral ischemia

Ischemic stroke (IS) is associated with a pronounced reduction in glucose and oxygen (O_2) supply to cerebral tissue due to major arterial occlusion. Due to improper medical treatments, around millions of neuronal cells in the brain can die quickly as a consequence of excitotoxicity-mediated brain injury due to excessive glutamate release that excites neurons to death via induced overproduction of free radicals and massive inflammation generated from recruiting leukocytes and activated microglia cells. However, the acute inflammation and oxidative stress that accompanies the early stages of stroke can result in the activation of detrimental transcription factors (e.g., Nuclear factor kappa-B (NF-kB)) to disrupt the blood–brain barrier, a physical barrier within the brain providing the protection and regulation of homeostasis. It is well understood that brain glucose metabolism and *(Narne et al., 2017)* blood flow serves as *bona fide* indices of neuronal synaptic activity. Hence, a disruption in these activities explicitly correlates with an impaired synaptic transmission as observed in the hypoperfused*(Hofmeijer et.al,,2012; vanPutten et al.,, 2012)* and electrically silent ischemic penumbra.

Mitochondria constitute the hub of metabolic networks, owing to their central role in the generation of ATP and reducing power(NADH, FADH2), anaplerosis, Ca2+ homeostasis and free radical detoxification. In line with this, a series of metabolic disruptions occur during the CI/ R in the highly dynamic mitochondrial compartment of neurons following oxygen and glucose deprivation (OGD) and glutamate*(Nicholls et al., 2009)*induced excitotoxicity. It induce discernible changes in the levels endogenous metabolites and energy intermediates of core pathways of intermediary metabolism (Villa et al., 2013). Mitochondria are quantifiable sources of reactive oxygen species (ROS), alteration in metabolic fluxes aggravates existing oxidative and nitrosamine stress that befits bioenergetic failure, at the same time antioxidant defence goes awry due to an overwhelming production of ROS and reactive nitrogen species (RNS). These perturbations impinge on cellular transcriptome and elicit an adaptive cellular phenotype CI/R Evidence indicates that inflammation*(Martinez B et al.,2017)* and immune response plays an important role in the outcome of ischemic stroke. Inflammation after stroke

involves leukocyte infiltration in brain parenchyma that contributes to cerebral damage. Peripherally derived mononuclear phagocytes, T lymphocytes, natural killer (NK) cells, and polymorph nuclear leukocytes, which produce and secrete cytokines, can all contribute to central nervous system (CNS) inflammation and gliosis Blood-derived leukocytes and resident microglia are the more activated inflammatory cells, accumulating in the brain tissue after cerebral ischemia, leading to inflammatory injury. Microglia, the major source of cytokines and other immune molecules of the CNS, are the first non-neuronal cells that respond to CNS injury, becoming phagocytic when fully activated by neuronal death. As cerebral inflammation is one of the earliest events in stroke, early intervention to modify the immune response may have a beneficial effect.

ROLE OF GSK-3ß IN ISCHEMIC STROKE.

Glycogen synthase kinase 3 beta (GSK-3β) has multiple functions, including regulating cellular development *(Guo et al.,2014; Jiang et al.,2014)* and tissue protection. GSK-3β is constitutively active in cells and its inactivation could be triggered by phosphorylation at Ser-9 Growing evidence implicated that GSK-3β is involved in brain tissue protection against ischemic injury or brain trauma. A selective GSK-3β inhibitor could cause the phosphorylation at Ser-9 and inactivate GSK-3β, thus provided a protective effect against transient cerebral ischemic injury. The inhibition of GSK-3β was effective for regulating metabolism disorders. Moreover, acute GSK-3β inhibition was proved to be a novel therapeutic strategy for acute myocardial infarction in a diabetic model. Thus, the GSK-3ß signalling pathway might be involved in cerebral ischemic injury in DM.

Cerebral ischemic stroke arises due to the blockade of cerebral artery which involves in supplying blood to this brain region. Due to this*(Darshit et al.,2017)*blockade, massive reduction of cerebral blood flow leads to reduced oxygen and glucose delivery and hence leads to energy deprivation as well as accumulation of toxic metabolites. Nerve cells that do not store energy source, which is lacking in ischemia will lead to multiple initial consequences like metabolic stress, failure in energy production, and ionic transport dysfunction (both ATP dependent and independent). Vulnerability after ischemic insult depends on duration of insult, as the neurons die within a minute of severe exposure. Depending on duration of ischemic insult, focal or global as well as transient or permanent, several signalling pathways getting into act to alter the neuronal morphology. In the initial

phase of insult, defense mechanisms get activated and try to normalize the effect. During the subsequent phases, apoptosis signalling determines the cell fate.

The time dependent change in gene expression (374 genes) of different pathways related to ischemia was assessed and shown to have a consistent change in Tumour necrotic factor receptor (TNFR) signalling pathway in all time intervals. Apart from TNFR signalling pathway genes, protein tyrosine kinase 2 (PyK2), protein kinase C (PKC), E2F transcription factor 1 (E2F1), vascular endothelial growth factor-A (VEGF-A), and many others were also changed at different time intervals. There were significant morphological alteration and infract volume found prominent at 12 and 24 HR, rather than at 3 hr. Several studies showed importance of reactive oxygen species (ROS) during initial phase in ischemia and its involvement in cellular signalling pathways.

There is sufficient evidence suggesting involvement of GSK-3ß in cerebral ischemia. Research reveals that over activation of GSK-3 β (Tyr216 phosphorylation) was found directly correlated to cerebral ischemia. Further, lithium induced GSK-3ß inhibition showed protective mechanism in cerebral ischemia. This observation suggests that the reduction of GSK-3ß activity improves brain cell survival. Activity of lithium in cerebral ischemia suggests that the PI3K/AKT/GSK-3ß link correlates in cerebral ischemia. Other studies involves inhibition of GSK- 3ß inhibition by Chir025 showed neuroprotective effect by reducing infract size, Bax, caspase-3 and -9 activities both in *in vitro* and *in vivo* models of cerebral ischemia, suggesting role of GSK-3ßinvolved in the mitochondrial pathway in pathology. Research revealed the role of GSK-3ß inhibition by NP00111, AR-A 014418, and lithium in hippocampal neuroprotection through ERK and PPAR receptor signalling pathway in oxygen-glucose deprivation. This observation highlighted the link between GSK-3ß, ERK, and PPAR in cerebral ischemia. A study relating multiple pathways like calcium signaling (S100B - S100 calcium binding protein B) kinase pathway (MAPK/JNK-1, -2/p38), inflammatory pathway (NF-(kappa) ß, TNF-alpha, COX-2, iNOS and ICAM-1), and typical mitochondrial apoptosis pathway (SOD, cytochrome c caspase-9 and Bcl-2) were shown to have direct relation with GSK-3ß in cerebral ischemia. Inhibition of GSK-3ß showed neuroprotection through down-regulation of these multiple pathways.

Activation of GSK-3ß can also induce apoptosis by caspase independent pathway involving p53 - a transcription factor which can regulate signalling dependent transcription Of genes. GSK-3ß forms a complex with nuclear p53 through binding to c-terminal region and phosphorylates Ser33, Ser315, and Ser376 residues. Upon interaction ofGSK-3ß with p53, short lived p53 will stabilize by variety of post translational modification such as phosphorylation, methylation, glycosylation, acetylation, sumoylation, and ubiquitination. GSK-3ß regulates p53 by phosphorylation of p53 specific E3 ubiquity ligase MDM2. In stroke, GSK-3*β*has been reported to be involved in neuronal cell death, whereas treatment with GSK-3*β*inhibitors in the acute state reduces infarction volume and improves neurobehavioral function. In the chronic state,GSK-3*β*inhibitors promote neurovascular remodelling after stroke and improve post ischemic stroke sequel.

It have been reported that inhibition of glycogen synthase kinase-3 (GSK-3) activities is neuroprotective against ischemic stroke. The kinase activities of GSK-3 are negatively regulated by phosphorylation*(Chern et al.,2014; Wang et al.,2014)* of GSK-3a at Ser21 and/or GSK-3b at Ser9. GSK-3 can be inhibited through direct binding to the ATP dependent magnesium-sensitive catalytic site of the enzyme and/or indirectly through enhanced serine phosphorylation of GSK-3 iso forms by multiple mechanisms. Inhibition of GSK3 rescues not only neurogenesis, but also hippocampal depending learning. Conversely, increased GSK3 activity contributes to generate a neuroinflammatory environment and also impairs adult neurogenesis. These results support the idea that inhibition of GSK3 is a potential target for treating ischemic stroke.

NEUROINFLAMMATORY PATHWAYS IN ISCHEMIC STROKE

Inflammation plays an vital role in the pathogenesis of ischemic stroke and other forms of ischemic *(Jin et al.,2013)* brain injury. Cerebral ischemia triggers a series of proinflammatory molecular and cellular events, such as rapid activation of resident cells, infiltration of various types of inflammatory cells into the ischemic brain tissue, and production of proinflammatory mediators, including cytokines and chemokines. Chemokines are small, inducible, secreted, proinflammatory cytokines that act primarily as chemo attractant sand activators of granulocytes, macrophages, and other inflammatory cells. Chemokines constitute a large family of*(Kim et al.,2014)* structurally related cytokines. Chemokines belong to a rapidly expanding family of cytokines. Their primary function is to

control the positioning of cells in tissues and to recruit leukocytes to the site of inflammation. Some of the study reported that levels of a variety of chemokines such as monocyte chemoattractantprotein-1 (MCP-1) increased in animal models of ischemia and patients with stroke.

LINK OF GSK-3ß AND INFLAMMATION IN STROKE

GSK-3 acts as a regulator of apoptosis and inflammation, known contributors to stroke-induced cell death *(Venna et al,2015:Benashsklet al.,2015)* Loss of GSK-3b, not GSK-3a, suppressed spontaneous neuronal death in extended culture models. Nonselective GSK-3b inhibition with lithium is neuroprotective and GSK-3b inhibitors are currently being tested in clinical trials for treatment of cognitive deficits and dementia. GSK-3beta is known to interact with the mitogen-activated protein kinase family (MAPKs) and promotes signalling after stress. Transforming growth factor-b-activated kinase-1 (TAK1) is a member of the MAPK family that is also known as mitogen-activated protein kinase kinase kinase-7. TAK1 is activated by TGF-b, tumour necrosis factor-a (TNF-a), and other cytokines including interleukin-1 (IL-1). TAK is also an upstream kinase of 5′ adenosine monophosphate-activated protein kinase (AMPK), a key energy sensing kinase involved in stroke. We found that inhibition of TAK1 is neuroprotective after focal ischemia (White et al. 2012). Our previous work demonstrated that neuroprotective effects of TAK1 inhibition are independent of its activation of AMPK.

GSK-3ß IN RELATION WITH MICROGLIA ACTIVATION AND NEUROINFLAMMATION

Proinflammatory cytokines are major central functioning molecules responsible for neurodegenerative disorders including PD. Microglia are the one which recruits toxic cytokines and*(Darshit et al.,2017)* other pro inflammatory mediators in the brain during pathology and responsible for neuronal loss. Both animal models and human PD patients showed activated microglia in SN brain region. Activated microglia up-regulates number of pro inflammatory cytokines like IL-1ß, IL-2, IL-6, TNF-ð, as well as transcription factor NF-κß which in turn up-regulates various other genes responsible for propagation of PD pathology. Suppression of inflammatory response. Through activated microglia showed beneficial effect against toxic challenge in PD pathology. Inflammatory response activated by

microglia is regulated by GSK-3ß.GSK-3ß acts as a centre bridge in a wide spectrum of signalling mechanism. Pro inflammatory cytokine production is enhanced from activated microglia by activation of GSK-3ß. Apart from cytokine production, GSK-3ß also promotes apoptosis through caspase dependent (caspase-3) and independent (p53) mechanisms, NF-κß mediated transcription, and several other signalling pathways discussed earlier to enhance progressive neuronal loss in PD. Inhibition of GSK-3ß can reverse neuroinflammation mediated through activated microglia. The anti inflammatory effect of GSK-3ß seems to be mediated through downregulation of a major pro inflammatory marker like TNF-ά. Other proposed mechanism of the neuroprotective effect of GSK-3ß is through NF-κß, PI3K/Akt/JNK signalling pathways.

ROLE OF GSK-3ß AND APOPTOSIS IN ISCHEMIA

GSK-3 acts as a regulator of apoptosis and inflammation, known contributors to stroke-induced cell death. Glycogen synthase kinase 3β which is inhibited by activating Akt, hence making it a key target of thePI3K/Akt survival signalling pathway GSK-3β is involved in multiple cellular processes; GSK-3β inhibition reduces infarct size in adult stroke models and further interacted with pro-apoptotic transcription factors, such as p53, which upregulates cytochrome C release and Caspase-3 activity providing more evidence of GSK-3β's pro-apoptotic role following brain injury .Glycogen synthase kinase (GSK-3β), which participates in a myriad of pathways throughout the central nervous system,(*Li L et al.,2015; Doycheva et al.,2015)* can be either neuroprotective or neurodegenerative depending on the site of phosphorylation; GSK-3β activity is increased by phosphorylation of tyrosine-216 and decreased by serine-9 phosphorylation While there is substantial evidence that GSK-3β inhibition reduces neuronal apoptosis experimental evidence of GSK-3β's effects6 on BBB stabilization and attenuation of inflammation are limited.

Mitochondria serve as a mechanical tool for indication of ROS and subsequent signalling Cytochrome c, a mitochondrial protein and component of respiratory chain, is an intermediate in mitochondrial mediated cell death signaling. Cytochrome c gets released from mitochondria into the cytosol and activates caspase proteins for apoptosis of neurons, which are found in hippocampal CA1 region subjected to transient focal cerebral ischemia. Released cytochrome c forms apoptosome by interacting with apoptosis activating factor 1 (Apaf1), cell death protein-4 (CED-4), and dATP [107, 108]. This apoptosome activates caspase-9

which initiates caspase cascade by activating caspase-3, -2, -6, -8 and -10. Caspase activated DNAse (CAD), which is activated by caspase-3, then starts fragmenting \DNA. Apart from CAD, activation of caspase cascade leads cleavage of Poly (ADP ribose) polymerase(PARP), DNA fragmentation, and apoptosis. Function of PARP is dependent on NAD and can cause depletionin PARP overproduction, which in turn can deplete ATP stores and leads to energy failure. Apart from cytochrome-C, a second mitochondria-derived activator of caspase (Smac) also takes part in caspase cascade. Unlike cytochrome c and Smac, inhibitor of apoptosis protein (IAP). Suppresses apoptosis by preventing the cleavage of procaspase and inhibits caspase activity. Activity of IAP is inhibited by Smac which binds to IAP and promotes caspase-3 activity.

There are other mediators that coincide in apoptosis like the Bcl-2 family proteins, which moderate apoptotic signalling by regulating mitochondrial membrane permeability. Studies also demonstrated that B cell lymphoma-2 (Bcl-2) family proteins regulate permeability transition pore (PTP) that is believed to release cytochrome c from mitochondria. Among Bcl-2 family proteins, Bcl-2 associated X protein (Bax), Bcl-XS, Bcl-2 homologues antagonist killer (Bak), BH3 interacting-domain death agonist (Bid), and Bcl-2 associated death promoter (Bad) are proapoptotic and facilitate cytochrome c release through PTP. Whereas, Bcl-2, and Bcl-XL have antiapoptotic activity, which blocks the release of cytochrome-c from mitochondria. Another neurotoxic material in cerebral ischemia is glutamate excitotoxicity. It is evident that elevated level of glutamate found in brain after cerebral ischemia leads to over stimulation of N-methyl D-aspartate receptor (NMDAR), which drives excitotoxic neuronal cell death. At the beginning phase of cerebral ischemia, the lack of oxygen and glucose leads to energy deprivation and subsequent failure of ATP dependent ion pumps. Following this alteration of electrolyte homeostasis, glutamate transport will be arrested. Hence accumulation of excess glutamate in extracellular compartment will over stimulate NMDAR and the further increase of calcium influx results in calcium mediated signalling by activating several intracellular enzymes and ultimate failure of mitochondria, generation of ROS, neuroinflammation, and apoptosis. Cerebral ischemia also activates other signalling pathways like p53, JNK \, c-jun, p38, CDK5, calpain, mTOR, hedgehog, notch, ERK-1, -2, PI3K/AKT/GSK-3, and many others. Upon activation of those pathways, downstream signalling will further propagate apoptosis

Hydrogen sulfide enhances N-methyl-D-aspartate receptor (NMDAR) -mediated responses, and facilitates the induction of the long-term potentiation of hippocampal neurons. However, cell culture*(Dai1 et al.,2015; Xu1 et al.,2015)* experiments have shown that high concentrations of hydrogen sulfide induce proapoptotic processes in hippocampal neurons by modulating the excitability of NMDARs. These findings of the effects of hydrogen sulfide on hippocampal neurons can be explained by the selective modulation of the NMDAR subunits, NR2A and NR2B. The activation of NR2A on synaptic neurons promotes the survival of hippocampal neurons via cAMP response element-binding protein (CREB) and phosphatidylinositol 3 -kinase (PI3K) -Akt signaling pathways, whereas the activation of NR2B on extrasynaptic neurons promotes neuronal apoptosis via the downregulation of CREB and the activation neuronal nitric oxide synthase (nNOS) - and JUN-mediated gene expression. The phosphorylation of the Akt serine-threonine kinase (also known as protein kinase B), a downstream target of PI3K, upregulates the phosphorylation of BAD, caspase-9, NF-κB, and Gsk-3β, which inhibits neuronal apoptosis.

GSK-3ß IN RELATION TO MITOCHONDRIAL DYSFUNCTION

Mitochondria are an important organelle for energy generation through ETC. Complex-I is involved in oxidative phosphorylation process and is the main site for the generation Of ROS through electron transfer to oxygen, generating O_2-, and H_2O_2 during oxidative stress in PD. In one of the models of PD pathology, MPTP is metabolized by mono amino oxygenase B (MAOB) into a toxic substance named 1-methyl-4-phenylpyridinium (MPP+), which is then selectively taken up by the dopamine transporter.MPP+ accumulates in mitochondria and generates ROS by inhibiting complex-I and the destruction of dopaminergic neurons. GSK-3ß is found more active in mitochondria than cytosol and initiates mitochondrial dysfunction. Functioning of GSK-3ß in mitochondria is not understood properly. However, research suggests that GSK-3ß controls ROS production through mitochondrial complex-I and regulates cell survival, and during pathology (MPTP and rotenone),are increased GSK-3ß activity blocks the complex-I activity and subsequent ROS production in SN dopaminergic neurons. Inhibition of GSK-3ß activity in MPTP and rotenone model protects dopaminergic neurons through recovering mitochondrial functioning and subsequent suppression of ROS production

GSK INVOLVED IN DIFFERENT DISEASE

GSK-3ß dysregulation contributes to the pathogenesis of many disorders, such as several cancers, diabetes, (*Avrahami et al.,2013: Licht-Murava et al.,2013)* and neuroinflammatory, neurodegenerative diseases. Neuroinflammation defined as a highly regulated biological response to harmful stimuli such as infectious agents and tissue injury is characterized by increased glial activation, pro-inflammatory cytokine concentration, blood–brain-barrier permeability, and leukocyte invasion.

Numerous studies have now implicated GSK-3 in control of various neuronal functions and have demonstrated that aberrant regulation of GSK-3 is involved in the etiology of neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Alzheimer's disease (AD), as well as in brain aging. Transgenic mice expressing elevated GSK-3 activity display memory deficits, reduced brain size and alterations in mood behaviour and social interactions. Abnormal regulation of GSK-3 activity is reported in patients with AD, ALS, major depression, schizophrenia and bipolar disorder. The mechanisms linking GSK-3 with pathogenesis likely involve regulation of targets that are directly or indirectly controlled by GSK-3. Thus, 'normalization' of GSK-3 activity emerges as a promising therapy for treatment of neurodegenerative and behaviour disorders. Inhibition of GSK-3 results in beneficial outcomes in multiple in vivo models.

Neurotherapeutic effects of GSK-3 inhibition in invivo animal models or patients.

TABLE:1

Neuro pathology	**Biological activity of GSK-3 inhibition**	**Animal models**
Alzheimer's disease (AD)	Ameliorates Aβ pathology Reduces tau phosphorylation Improves learning and memory Attenuates inflammation Neuroprotective	FTDP-17 tau transgenic mice APP/PS1 mice hAPP mice Rats injected with preformed β-amyloid hTau (T44) mice 3xTg mice GSK-3β mice Postnatal rats

	activity	
Amyotrophic lateral sclerosis (ALS)	Attenuates motor neuron death Induces clearance of protein aggregates Improves learning and memory	Human ALS patients G93A-SOD1 mice
Bipolar disorder (BD) and Depression	Anti-depressive activity Neuroprotective activity Reduces the prevalence of AD Mood stabilizer (Lithium salts)	Human BD patients "Clock mutant" mice- hyperactivity induced by amphetamine/chlorodiazepoxide/methamphetamine
Cerebral ischemia (CI)	Attenuates neuronal death Reduces infraction size Reduces inflammation Neuroprotective activity Improves behavioral functions	Ischemic stroke rat Rats subjected to permanent middle cerebral artery occlusion Rats with hypoxia–ischemia brain injury
Epilepsy	Reduces the development of hippocampal Sclerosis (lithium salts)	Rats injected with kainite
Fragile X	Reduces audiogenic	Mice with mutated Fmr1 protein

syndrome (FXS)	seizures frequency Improves learning and memory Improves behavioral functions Neuroprotective activity	Drosophila with mutated FMR1 protein
Huntington's disease (HD	Improves motor performance Reduces polyglutamine toxicity Neuroprotective activity	R6/2 mice (expressing mutated huntingtin) Drosophila expressing mutated huntingtin
Neuropathic pain	Anti hyperalgesic activity	Mice with partial ligation of the sciatic nerve (PSNL
Parkinson's disease (PD)	Promotes dopamine neurons differentiation	Mice treated with neurotoxin MPTP
Schizophrenia	Neuroprotective activity	ce treated with stereotoxic injection
Spinal muscular atrophy (SMA)	Maintains motor neuronal survival	Fibroblasts derived from SMA patients
Spinocerebellar Ataxia type 1 (SCA1)	Improves motor coordination Improves learning and memory	SCA1 (154Q/2Q) mice
Traumatic brain injury (TBI)	Anti-depressive activity	Traumatic brain injury in mice

ARA014418:

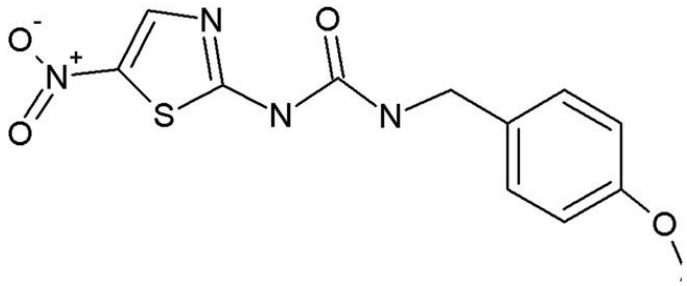


Figure.2.Chemical structure of ARA014418 (GSK-3β Inhibitor VIII CAS 487021-52-3)

TDZD8:

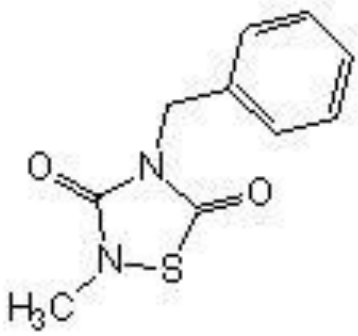


Figure.3.Chemical structure of TDZD8(GSK-3β Inhibitor I CAS 327036-89-5)

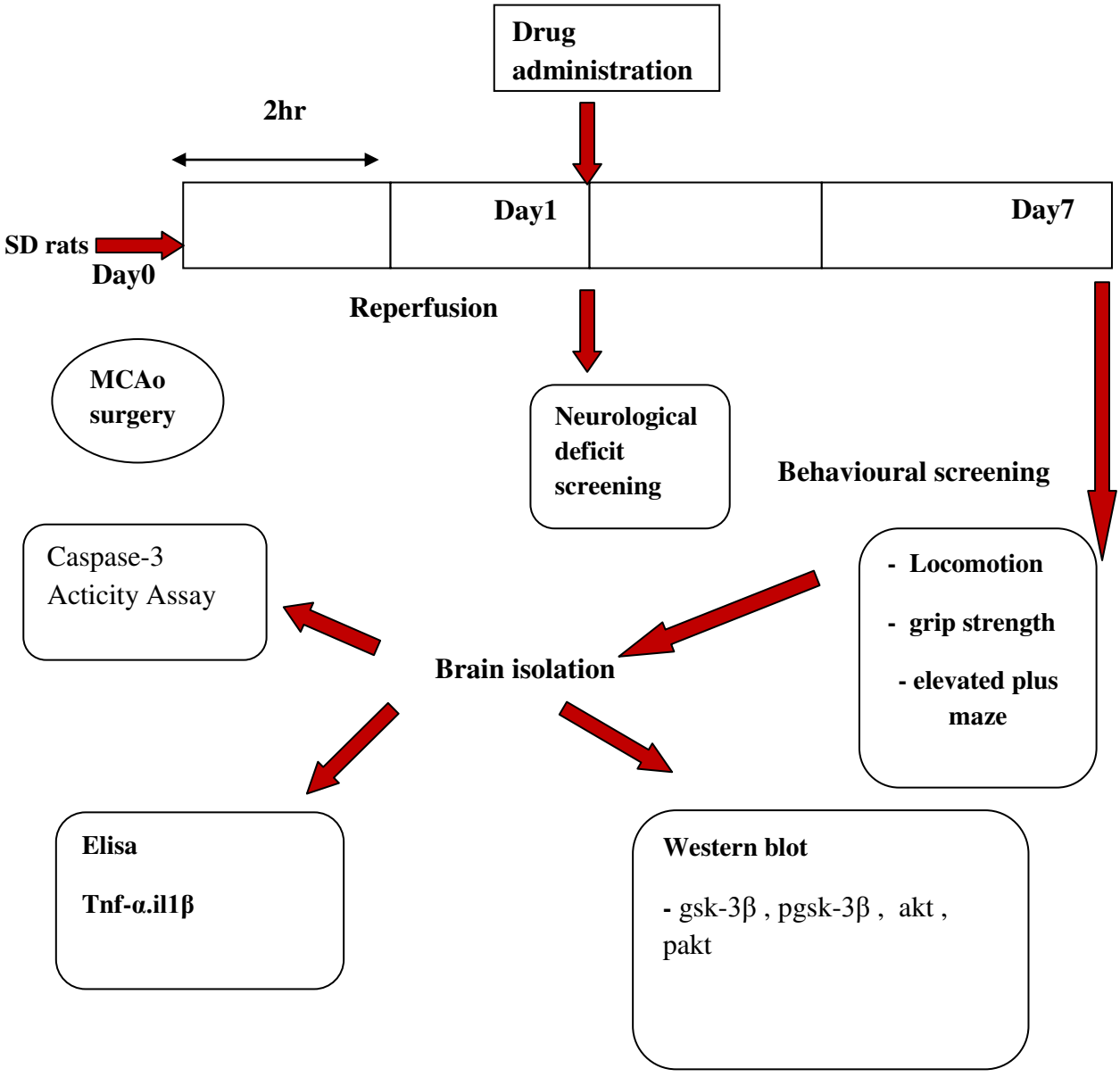
3.Objective & plan of study

3. OBJECTIVES & PLAN OF STUDY

The present study has been designed to address the following objectives

1. To study the effect of AR-A014418 And TDZD-8, GSK-3β inhibitor on cerebral ischemic condition by behavioural assessment.

2. To evaluate the neuroprotective activity mechanism of GSk3-β inhibitors in cerebral ischemia by exploring the anti-inflammatory pathways.



Phase-1

Inducing ischemia by MCAo (middle cerebral artery occlusion) model and drug treatments (*Güzel et al.,2014; Rölz et al.,2014)*

Phase-2

Assesment of GSK-3 β inhibition effect through measuring a neurobehavioral studies using following parameter.

2.1 Neurological deficit score (*Bederson et al., 1986*)

2.2 locomotor activity *(Borlongan et al.,1995; Cahill et al.,1995)*

2.3 Gripstength (*Gellman et al.,1989)*

2.4 Elevated Plus Maze *(Vécsei et al.,1990)*

Phase-3

3.1 Evaluation of anti-inflammatory mechanism of GSK-3 β inhibition through measuring-IL-1β, TNF-α.through ELISA.

3.2 Expression of protein measurement by Western blot.

GSK-3β , PGSK-3β , Akt , PAkt

3.3 Apoptotic pathway measurement through analysing Caspase -3 activity assay.

4.Materials & Method

4. MATERIALS & METHODS

Materials:

chemicals&tools used	Company name
ARA014418 (CAS 487021-52-3)	Santa Cruz
TDZD 8 (CAS 327036-89-5)	Santa Cruz
Crushing forceps	Finetools
Bonney forceps	Finetools
Adson tissue forceps	Finetools
Adson teeth forceps	Finetools
Horton adson forceps	Finetools
Retractors	Finetools
Scalpels	Finetools
Microvessel clips	Finetools
Scissors	Finetools
Silicon	Anabond
Caspase assay activity kit	Biovision inc
elisa kit	R&d systems
Temed	sigma-aldrich
Acrylamide/bis solution	Bio-red
Ammonium persulfate	sigma-aldrich
Peroxide solution	thermo scintific
Luminal enhancer solution	thermo scintific
Glycine	sigma-aldrich

Methods:

4.1. Animals

Male Sprague-Dawley rats (290-340) used in the study were housed in individual polypropylene cages in a well ventilated room (air cycle: 15/hr) under an ambient temperature of 23±2ºC and 40-65% RH, with a 12:12 h light/dark artificial photo period. They were provided with food and purified water . All the animals were acclimatized atleast for 7days to the laboratory conditions prior to experimentation. Guidelines of "guide for the care and use of laboratory animals " were strictly followed throughout the study. Institutional animal ethical committee (IAEC), PSG Institute of medical science and research, Coimbatore ,approved the study.

4.2. Surgical procedure

Focal cerebral ischemia was induced by middle cerebral artery occlusion with minor modifications. Rats were anesthetized with ketamine (90mg/kg) and xylazine (15mg/kg).The left common carotid artery was exposed at the level of external and internal carotid artery bifurcation. 4-0 nylon monofilament was used and its tip was made round headed by exposing it to flame. The Filament was coated with Anabond silicone gel and inserted into the external carotid artery and advanced to the internal carotid artery for a length of about 20–21 mm until a slight resistance was felt. On achieving occlusion, the filament was held in place with ligature and the external incision was sutured temporarily. After 2 h of ischemia the rats were anesthetized, suture was opened, the filament was pulled out and reperfusion in internal carotid artery was ensured visually. Throughout the surgical procedure, body temperature was measured by inserting a thermometric probe into the rectum of rat and it was maintained at 37±0.5 °C using thermostatically controlled heating blanket. Animals were then kept in a cage with a heating lamp, which maintained the cage temperature between 29±1 °C for another 1 h to counteract any possible hypothermic effect. In the sham-operated (SO) group, external carotid artery was surgically prepared for insertion of filament, but the filament was not inserted (*Babu and Ramanathan, 2009).*

Groups and treatments :

Table.2

Number	Group +	Treatment
1	MCAO +	AR-A 014418 30µm/kg
2	MCAO +	AR-A 014418 60µm/kg
3	MCAO +	TDZD dose 1500µm/kg

4.3. Neurobehavioral paradigms

4.3.1. Neurological deficit(*Bederson et al., 1986*)

Neurological deficit were assessed by following score pattern after 24 hrs of ischemic reperfusion (IR)

Neurological deficits (6-point scale)

- 0 = No neurological deficit
- 1 = Failure to extend left forepaw fully
- 2 = Circling behaviour
- 3 = Falling to the left

- 4 = No spontaneous walking with a depression
- 5 = Death

4.3.2. Loco motor activity

After 168 hrs (7 days) of IR, the rats will be placed in the open field apparatus for recording the locomotion. The locomotor activity will be measured using an open field test. The behavioural parameters measured in open field test were Rearing ,Grooming, No.of ambulations, Time spent central compartment.(*Borlongan et al.,1995; Cahill et al.,1995)*

4.3.3. Grip strength measurement

After 168hrs(7 days) of IR the experimental animals , grip strength test by using bioseb grip strength meter was used to study of neuromuscular functions by determining maximal peak force developed by rodent were measured in units grams.(*Gellman et al.,1989)*

4.3.4 Elevated plus maze

Elevated plus maze test

The elevated plus maze test (EPM) consisted of two open arms (35 × 5 cm) crossed with two closed arms (35 × 5 ×20 cm). The arms were connected together with a central square of 5 × 5 cm. The apparatus was elevated to the height of 50 cm in a dimly illuminated room. Animals were placed individually at the end of either of the open arms facing away from the central platform. The time taken by each animal to move from open arm to either of the closed arms was recorded. This duration of time was called transfer latency (TL). If the animal did not enter into any of the enclosed arms within 120 s, it was gently pushed into any of the enclosed arms and TLwas considered as 120 s. Later the animal was allowed to explore the plus maze for 5 min and send back to its home cage. *(Vécsei et al.,1990)*

4.4. Probable mechanism of neuroprotection

4.4.1 Western blot

Western Blot Analysis for Protein Detection Cells were lysed in RIPA buffer along with protease and phosphatise inhibitor cocktail. Samples were kept on ice for 15– 30 min followed by sonication and centrifugation at 12,000 rpm for 5 min. The supernatant solution was taken and assayed for total protein content by Bradford's method. Equal protein concentration (20 μg) was used for SDS polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and blocked with 5 % bovine serum albumin prepared in TBST buffer for 3 h. The blocked membrane was incubated with primary antibody against Akt1 (1:750), S473p-Akt1 (1:1000), GSK-3β (1:500), S9 p-GSK-3β (1:1000), β-catenin (1:500), and reference protein β-actin (1:1000) for 2 h at room temperature. Following that, goat anti-rabbit IgG-HRPand goat anti-mouse IgG1-HRP secondary antibodies were added to the membrane in separate procedures according to the primary antibody used in the previous step and incubated for 1 h at room temperature. The membrane was developed and detected by Gel Dock system, G: Box (Syngene, Frederick,MD, USA). Row volume was considered for analysis.

4.4.2GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well micro plate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 μL of reagent diluent to each well. Incubate at room temperature for a minimum of 1 hour.

4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in reagent diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.

3. Add 100 µL of the Detection Antibody, diluted in reagent diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4. Repeat the aspiration/wash as in step 2 of Plate Preparation.

5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Repeat the aspiration/wash as in step 2.

7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

4.4.3. Caspase activity assay

Cells were harvested and lysed in 50 µl of lysis buffer by incubating on ice for 10 min. Centrifugation at 10,000×g for 2 min was done to remove cell debris. The supernatant extract was taken, and protein measurement was done by Bradford's method. Total protein (100 µg) was used in 50 µl of cell lysis buffer. To this, 50 µl of 2× reaction buffer containing 10 mMDTT and 5 µl of 4 mM of DEVD-pNA (caspase-3 substrate) was added and incubated at

37 °C for 90 min. Absorbance was measured at 405 nm by Multiskan™ GO multiplate reader(Thermo Scientific, Waltham, USA). The fold change in enzyme activity was measured

5. Statistical Analysis

The data are expressed on mean ±SD.The results will be analysed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to assess the statistical significance of the differences among the study groups using Graph Pad Prism5.

5.Results

5. RESULTS

Phase-5.1: MCAo (Middle cerebral artery occlusion) surgery optimization

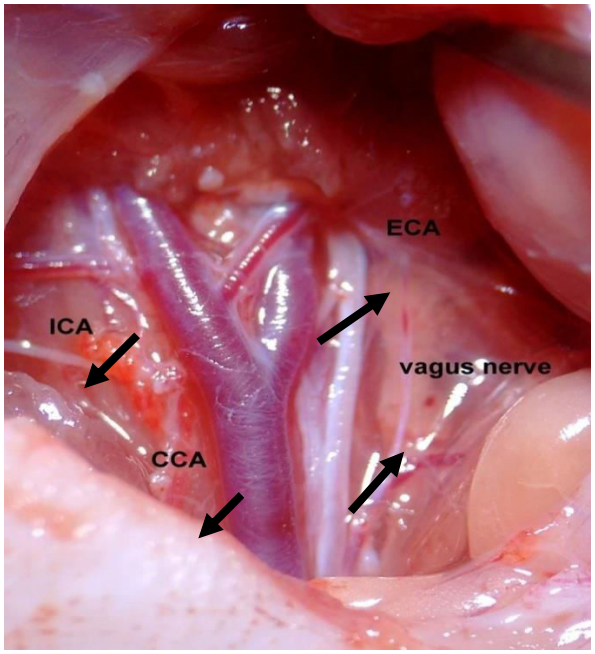


Figure.4: Branches of common carotid artery (CCA)

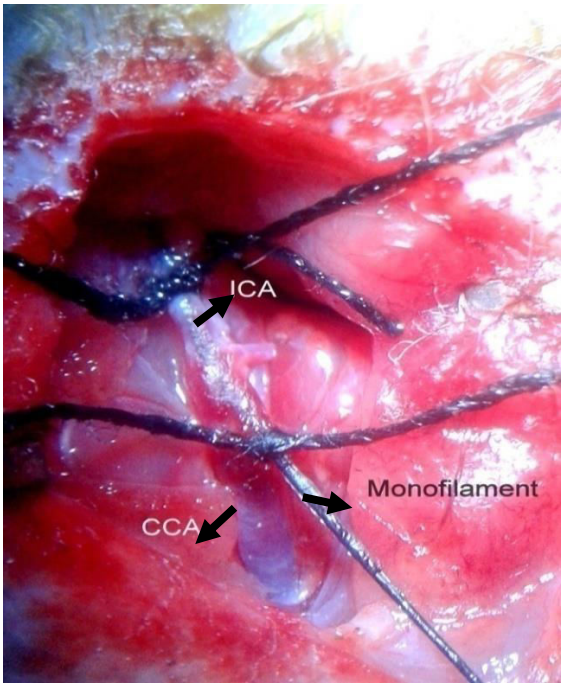


Figure .5: Insertion of the monofilament to ICA through ECA

PHASE5.2 BEHAVIOURAL STUDIES

5.2.1.Neurological deficit score:

The neurological deficit score was evaluated after 24hrs of IR and on 7th day. After 24hrs and on 7th day there was significant increase in neurological score in MCAo group ($p<0.01$) is comparison to control group. On 7thday, post traumatic treatment of TDZD-8 (1500µm/kg) only showed significant ($p<0.05$) decrease in neurological score in comparison to MCAo group.

5.2.2.ELEVATED PLUS MAZE:

5.2.2.1.Effect on time spent in central compartment

Data are expressed in seconds. MCAo group ($p<0.001$) showed significant decrease in central compartment exploration is comparison to control group indicating increase in anxiety in the rat. There was no significant difference between MCAo and treatment groups.In this parameter was observed.

5.2.2.2.Effect on no of entries in closed arm

The number of entries made in the closed arms was found to be statistical insignificant between groups.

5.2.2.3.Effect on no of entries in open arm

MCAo group ($p<0.05$) showed significant decrease in no of entries made in open arm is comparison to control group indicating increased anxiety of the rat. There was no significant difference between MCAo and treatment groups.

5.2.2.4.Effect on time spent in closed arm

Data one expressed in seconds. MCAo group showed significant ($p<0.001$) increase in time spent in closed arm when comparison to control group indicating increase in anxiety. AR-A (30µg/kg), $p<0.01$ and (60µm/kg),$p<0.001$ &TDZD-8(1500mg/kg),$p<0.001$ showed significant decrease in time spent in closed arms in comparison to MCAo group indicating the decrease in angiogenic behaviour.

5.2.2.5.Effect on time spent in open arm

Data are expressed in seconds. MCAo rats exhibited significant ($p<0.001$) anxiety behaviour is an evidently decrease in time spent in open arm is comparison to control rats. AR-A (30µm/kg), $p<0.01$and (60µm/kg) ,$p<0.001$ &TDZD-8 (1500µm/kg), ($p<0.001$) treatment significant increase in time spent in open arms is comparison to MCAo group indicating the reversed of anxiety with treatment.

5.2.3.GRIP STRENGTH:

Data are expressed in force (gms). MCAo rats ($p<0.001$) showed significant decrease in force applied on the grid when compared with control group indicaties decrease in muscle tone of the rat. AR-A(60µm/kg), $p<0.05$ &TDZD-8 (1500µm/kg) ,$p<0.01$ showed increased in muscle tone as observed by increased comparison to MCAo group indicates increase in muscle strength of rat.

5.2.4.OPEN FIELD TEST:

5.2.4.1Effect on central compartment exploration

The exploratory activity of different treatment group in central compartment of open field exploratory time have shown no significance difference.

5.2.4.2.Effect on ambulations

MCAo rats ($p<0.001$) showed significant decrease in number of ambulations when compared to control rats indicating reduced locomotor activity. AR-A (60µm/kg) and TDZD-8 (1500µm/kg) ,($p<0.01$) treatment exibited significant increase in ambulations in comparison to MCAo group indicaties improved locomotor activity.

5.2.4.3.Effect on grooming&rearing

The rearing &grooming behaviour of IR rats and different treatments showed no significant difference in open field tests.

5.2.4.5.Effect on licking

MCAo rats ($p<0.05$) showed significant decrease in number of licking behaviour is comparison to control group of the rat. There was no significant difference with the treatment groups in comparison to MCAo rats.

5.2.4.6.Effect on freezing

MCAo rats ($p<0.001$) showed significant increase in freezing time is comparison to vehicle treated group indicaties depression the rats. AR-A (60µm/kg) and TDZD-8 (1500µm/kg) , ($p<0.001$) showed significant decrease freezing behaviour in comparison to MCAo rats indicates lower anxiety.

PHASE.5.3.

5.3**.1.Effect on pro-Inflammatory mediator(TNFα) by ELISA**

Data are expressed in pg/mg protein. MCAo rats showed significant ($p<0.001$) increase in TNF -α levels when compared to vehicle treated rats indicating increased inflammatory activity. AR-A (60µm/kg), ($p<0.01$) and TDZD-8 (1500µm/kg) ,($p<0.05$) treatment resulted in significantly decreased in TNF -α levels in comparison to MCAo rats indicating alteration of inflammation response induced with MCAo.

5.3.2.Effect on pro-inflammatory mediator(IL 1β) by ELISA

Data are expressed in pg/mg protein. MCAo inducement in rats showed significant ($p<0.01$) increase in IL- 1β levels is comparison to control rats indicating increase in inflammatory activity .AR-A (60µm/kg), ($p<0.01$) and TDZD-8 (1500µm/kg) , ($p<0.01$) showed significantly reduced in IL- 1β levels in comparison to MCAo rats indicating decrease in inflammation similarly Significant decrease in caspase enzyme activity. was observed to MCAo rats indicating reduced cell death or apoptosis.

5.4.3.Effect of drug treatment on ratio of GSK- 3β & P GSK- 3β level

Data are expressed in ratio. MCAo rats ($p<0.05$) showed significant decrease in GSK- 3β:p gsk-3ß level in comparison to control rats. AR-A (30&60µm/kg) $p<0.001$ tratment rats expressed significant increase in GSK 3β:p gsk-3ß ratio is comparison to MCAo rats.

5.4.4.Effect of drug treatment on ratio of PAKT:AKT by western blot.

Data are expressed in ratio. MCAo rats exhibited significant ($p<0.05$) decrease in AKT:p AKT ratio in comparison to vehicle traeted rats. Treatment of AR-A (30µM/kg) $p<0.05$, (60µM/kg) ,$P<0.01$ showed significant increase in AKT:pAKT ratio in comparison to MCAo rats .

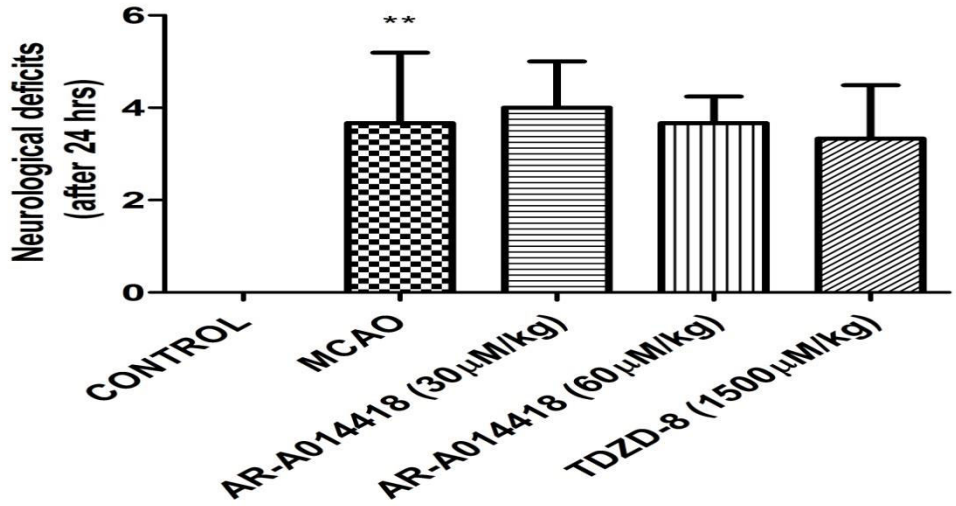
5.4.5Effect on caspase activity assay

Data are expressed as specific activity/µg protein. MCAo rats showed significant ($p<0.001$) increase in caspase enzyme activity in comparison to control rats indicating increase in apoptosis (cell death). AR-A (60µm/kg), $p<0.01$ and TDZD-8 (1500µm/kg) ,$p<0.05$ showed

Results

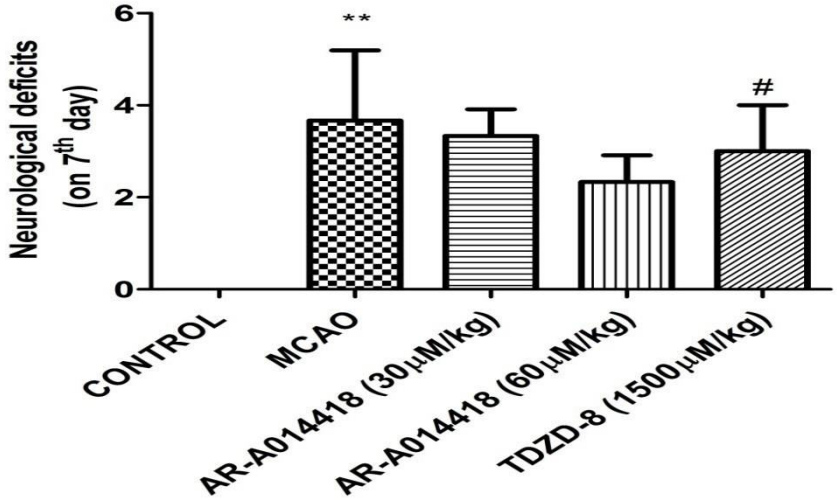
BEHAVIOURAL STUDIES:

Figure:6 Neurological deficit score after 24 hrs



Data are expressed as mean ±SD (n=6). **denotes statistical significance of MCAo group compared with control group at p<0.01.

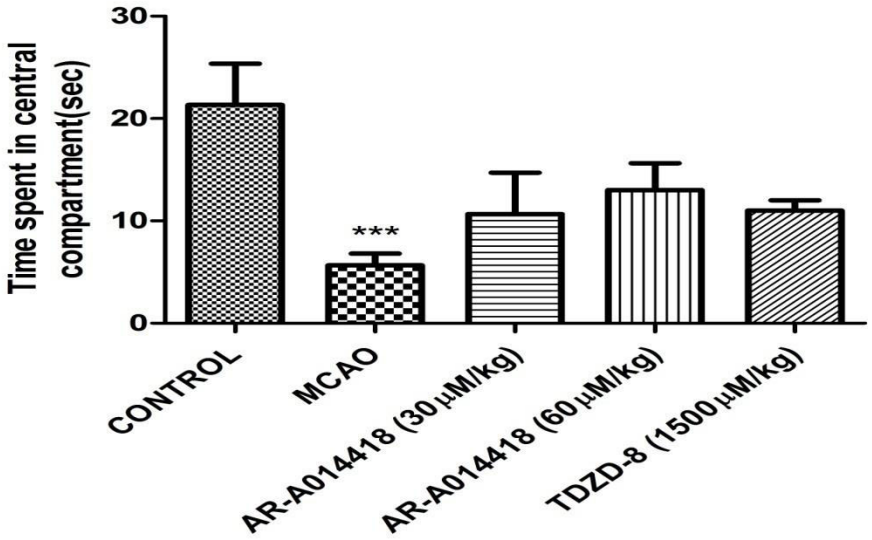
Figure.7. Neurological deficit score on 7th day



Data are expressed as mean ±SD (n=6). **denotes statistical significance of MCAo group compared with control group at p<0.01. #denotes statistical significance of TDZD-8 (1500µM/kg) treated group compared with MCAo group at p<0.05 respectively

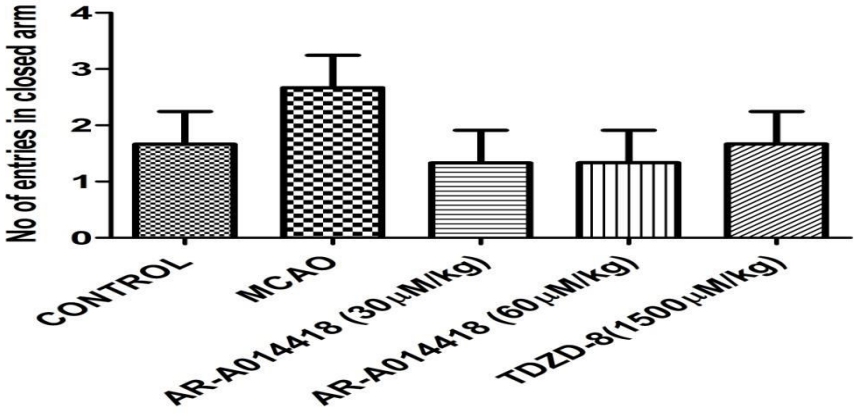
ELEVATED PLUS MAZE

Figure.8.Effect of drug treatment on time spent in central compartment



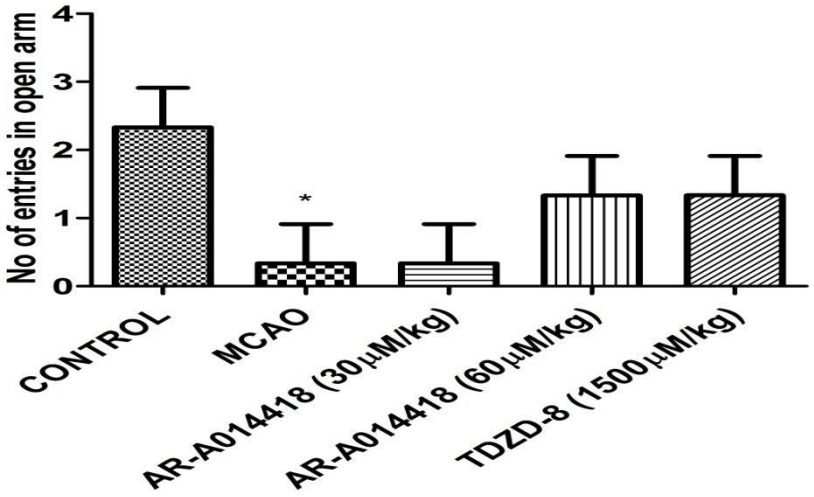
Data are expressed as mean ±SD (n=6). Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. *** denotes statistical significance of MCAo group compared with control group at $p<0.001$.

Figure.9.Effect of drug treatment on no of entries in closed arm



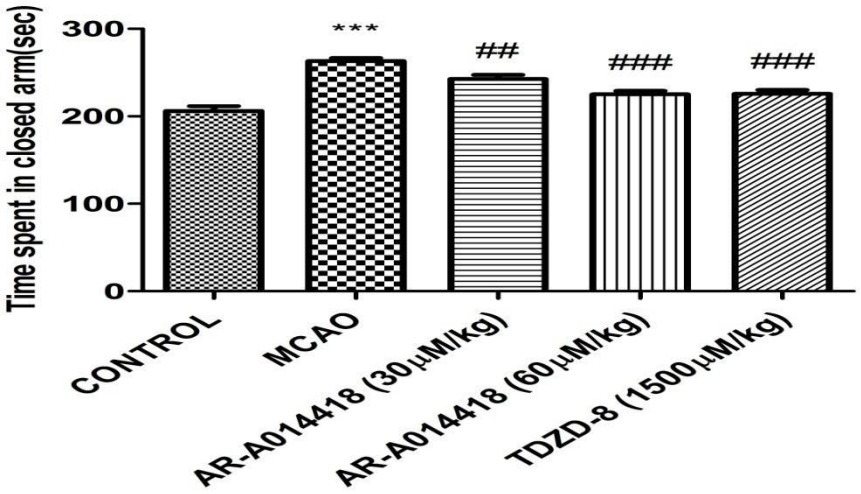
Data are expressed as mean ±SD (n=6). There is no statistical significance between the groups.

Figure .10.Effect of drug treatment on no of entries in open arm



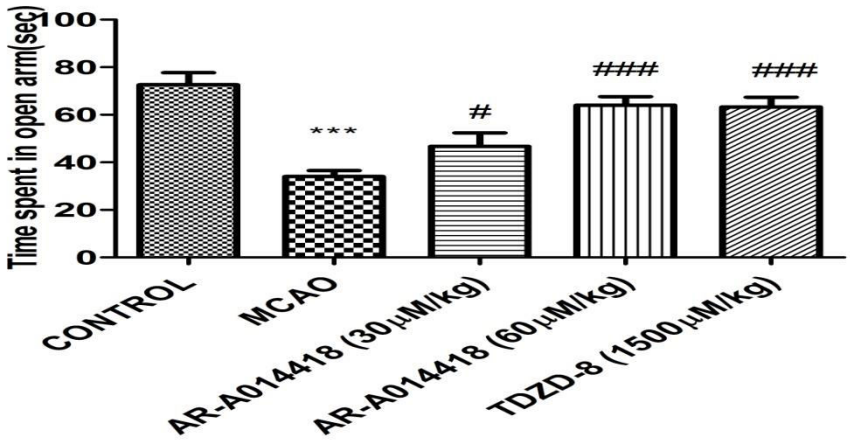
Data are expressed as mean ±SD (n=6). * denotes statistical significance of MCAo group compared with control group at $p<0.05$.

Figure.11.Effect of drug treatment on time spent in closed arm



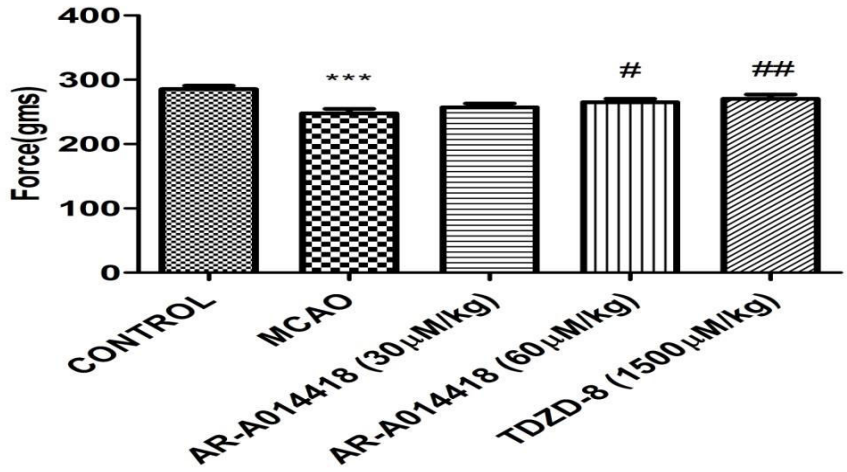
Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at $p<0.001$. ###,##,# denotes statistical significance of AR-A (30&60µM/kg), TDZD-8 (1500µM/kg) treated groups compared with MCAo group at $p<0.001$, $p<0.01$, $p<0.05$ respectively

Figure.12.Effect of drug treatment on time spent in open arm



Data are expressed as mean ±SD (n=6).. ***denotes statistical significance of MCAo group compared with control group at p<0.001.$^{###,##,#}$denotes statistical significance of AR-A(30&60µM/kg),TDZD-8(1500µM/kg) treated groups compared with MCAo group at p<0.001,p<0.01,p<0.05 respectively.

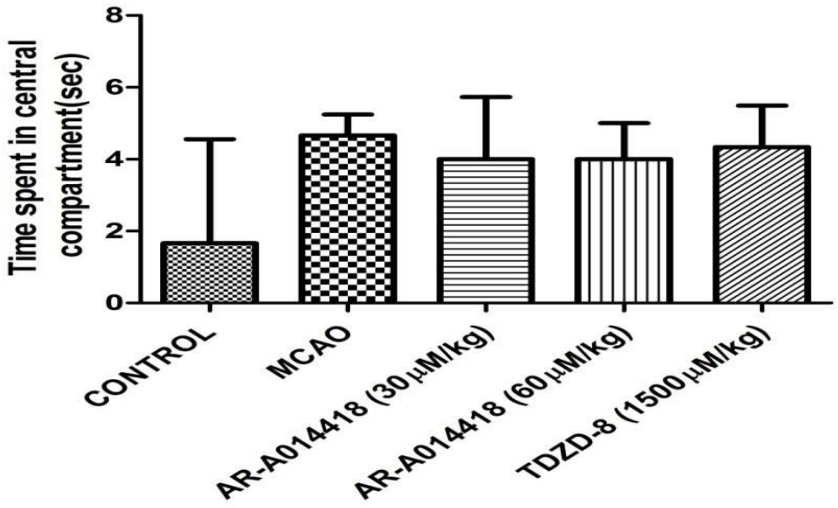
Figure.13.Effect of drug treatment on grip strength



Data are expressed as mean ±SD (n=6). ***denotes statistical significance of MCAo group compared with control group at p<0.001.$^{##,#}$denotes statistical significance of AR-A(60µM/kg),TDZD-8(1500µM/kg) treated groups compared with MCAo group at p<0.01,p<0.05 respectively.

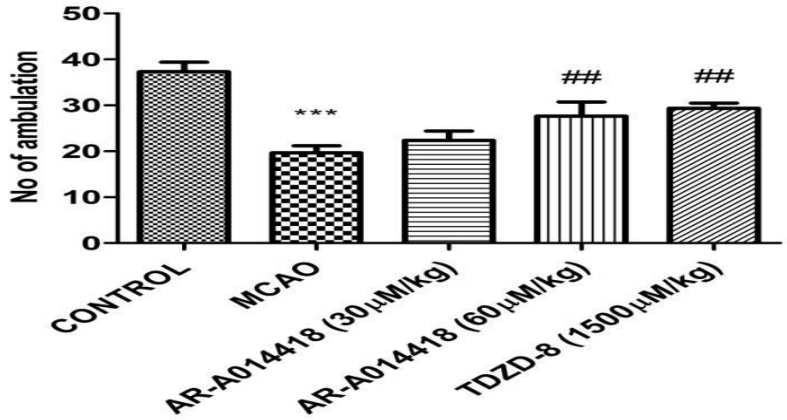
OPEN FIELD TEST

Figure.14.Effect of drug treatment on time spent in central compartment



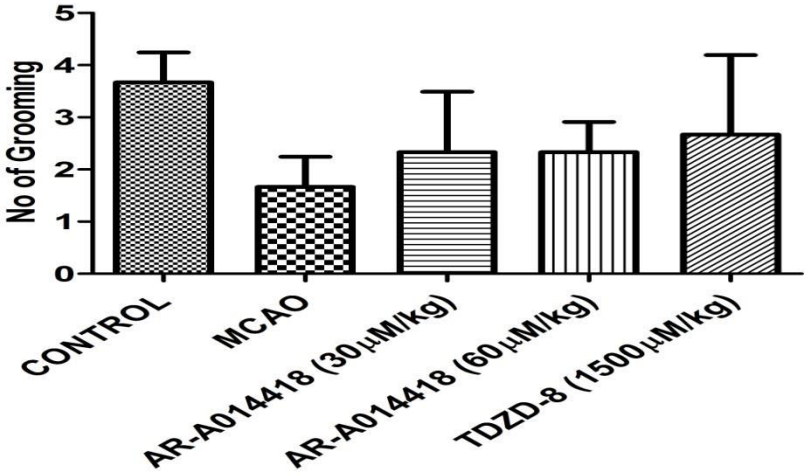
Data are expressed as mean ±SD (n=6). There is no statistical significance between the groups.

Figure.15.Effect of drug treatment on no of ambulations



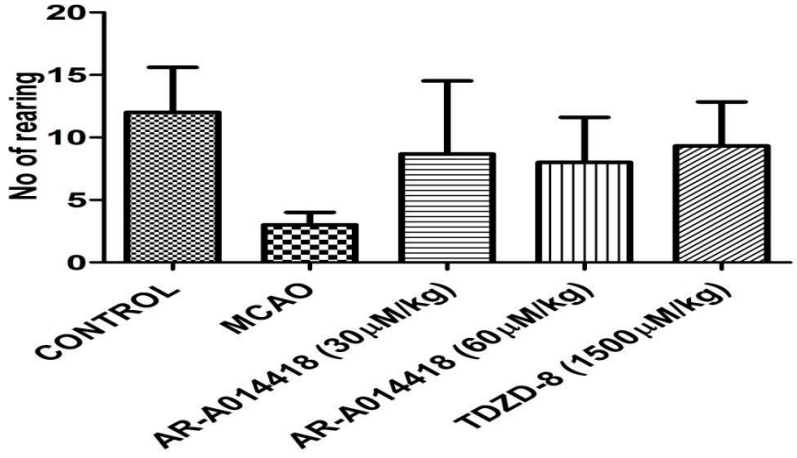
Data are expressed as mean ±SD (n=6). ***denotes statistical significance of MCAo group compared with control group at $p<0.001$.$^{##}$denotes statistical significance of AR-A(60µM/kg),TDZD-8(1500µM/kg) treated groups compared with MCAo group at $p<0.01$ respectively.

Figure.16.Effect of drug treatment on grooming



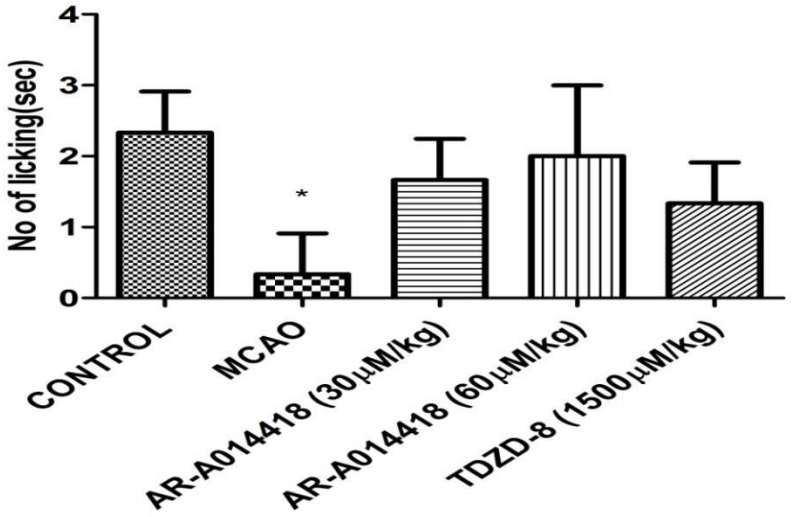
Data are expressed as mean ±SD (n=6). Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. There is no statistical significance between the groups.

Figure.17.Effect of drug treatment on rearing



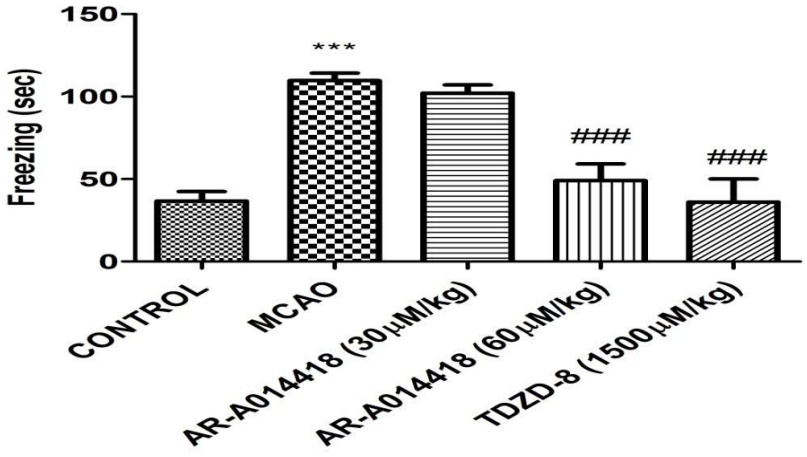
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. There is no statistical significance between the groups.

Figure.18.Effect of drug treatment on licking



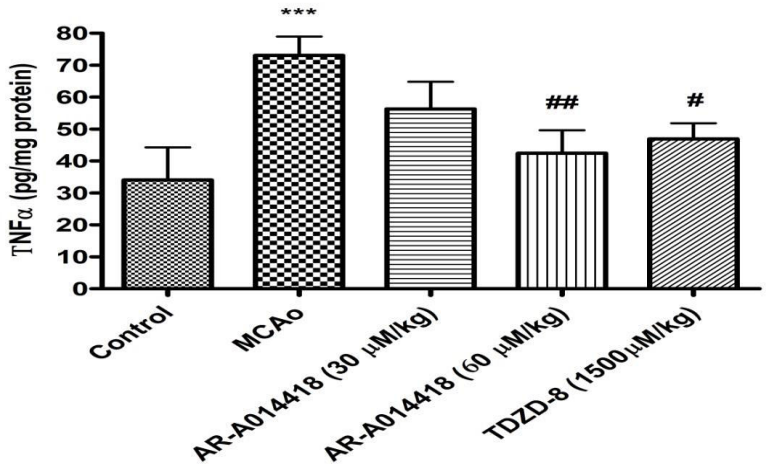
Data are expressed as mean ±SD (n=6). * denotes statistical significance of MCAo group compared with control group at $p<0.05$.

Figure.19.Effect of drug treatment on freezing



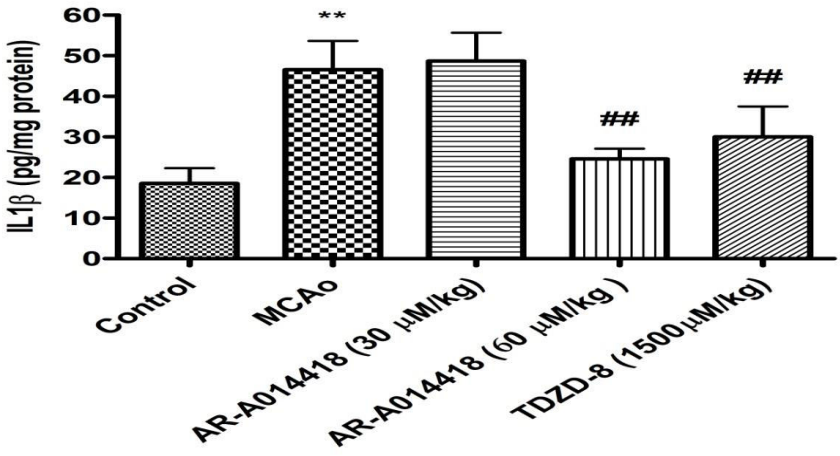
Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at $p<0.001$.### denotes statistical significance of AR-A (60µM/kg), TDZD-8 (1500µM/kg) treated groups compared with MCAo group at $p<0.001$ respectively.

Figure.20.Effect of drug treatment on pro-inflammatory mediator(TNFα) by ELISA



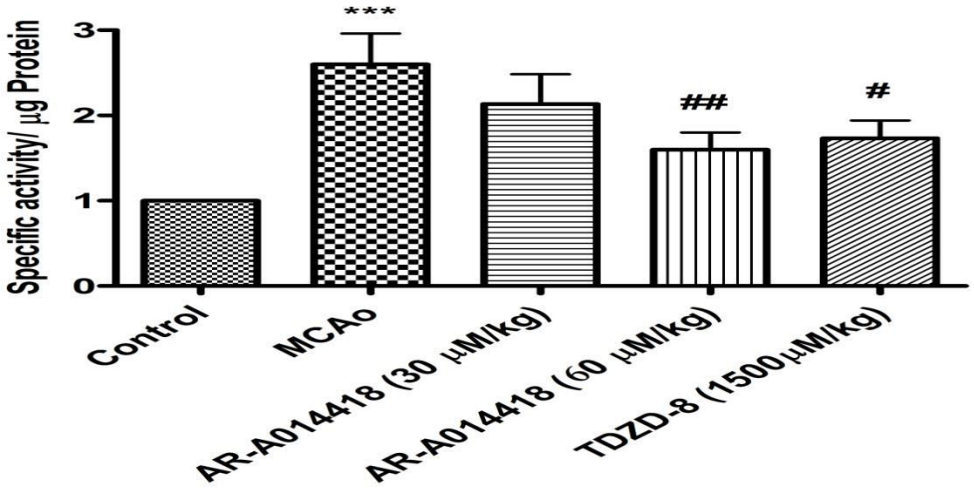
Data are expressed as mean ±SD (n=3). ***denotes statistical significance of MCAo group compared with control group at p<0.001.$^{##,\#}$denotes statistical significance of AR-A (60µM/kg), TDZD-8 (1500µM/kg) treated groups compared with MCAo group at p<0.01,p<0.05 respectively.

Figure.21.Effect of drug treatment on pro-inflammatory mediator(IL 1β) by ELISA



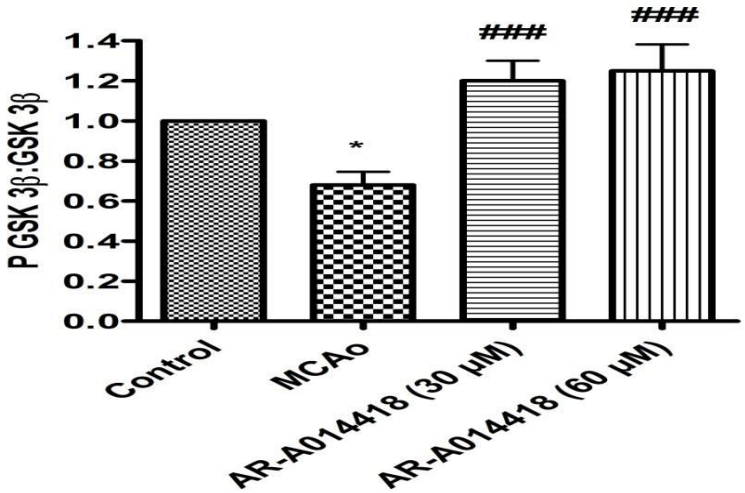
Data are expressed as mean ±SD (n=3). **denotes statistical significance of MCAo group compared with control group at p<0.01 $^{\cdot ##}$denotes statistical significance of AR-A (60µM/kg) ,TDZD-8 (1500µM/kg) treated groups compared with MCAo group at p<0.01 respectively.

Figure.22.Effect of drug treatment on caspase activity assay



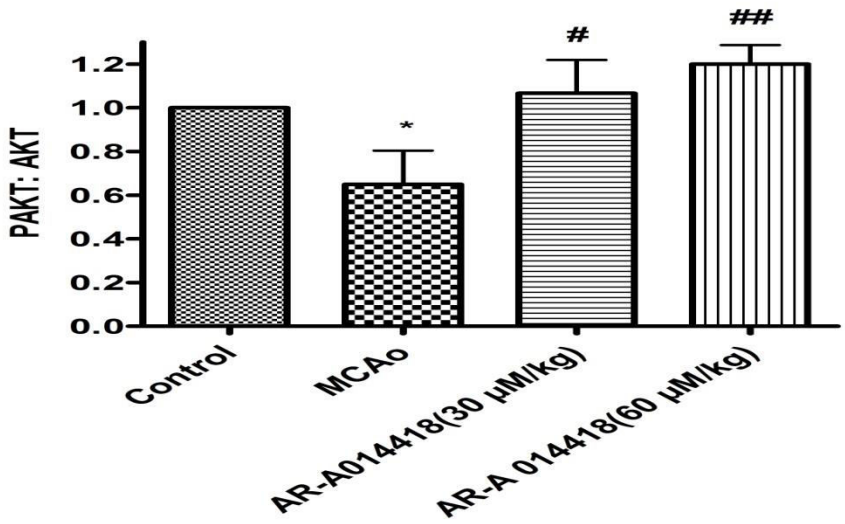
Data are expressed as mean ±SD (n=3).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. ***denotes statistical significance of MCAo group compared with control group at p<0.001 $^{.##,\#}$denotes statistical significance of AR-A (60µM/kg), TDZD-8 (1500µM/kg) treated groups compared with MCAo group at p<0.01,p<0.05 respectively.

Figure.23.Effect of drug treatment on ratio of GSK 3β & P GSK 3β western blot



Data are expressed as mean ±SD (n=3).. *denotes statistical significance of MCAo group compared with control group at p<0.05 .$^{###}$denotes statistical significance of AR-A(30 &60µM/kg) treated groups compared with MCAo group at p<0.001 respectively.

Figure.24.Effect of drug treatment on ratio of PAKT:AKT using western blot



Data are expressed as mean ±SD (n=3). *denotes statistical significance of MCAo group compared with control group at $p<0.05$. $^{\#\#,\#}$denotes statistical significance of AR-A (30&60µM/kg) treated groups compared with MCAo group at $p<0.01$,$P<0.05$ respectively.

FIGURE.25. Effect of drug AR-A014418(30&60µm/kg) on ratio of GSK-3β & pGSK-3 β

GSK-3β

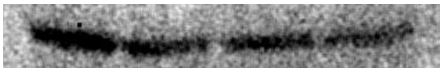
Control MCAo AR-A (30µm/kg) AR-A(60µm/kg)

pGSK-3β

Control MCAo AR-A(30µm/kg) AR-A(60µm/kg)

FIGURE.27. Effect of drug AR-A014418(30&60µm/kg) on ratio of AKT and pAKT

AKT



Control MCAo AR-A(30µm/kg) AR-A(60µm/kg)

pAKT

Control MCAo AR-A(30µm/kg) AR-A(60µm/kg)

6.Discussion

6.DISCUSSION

The objective of present study is to evaluate the neuroprotective role of GSK-3β inhibitor through supressing neuroinflammation in middle cerebral artery occlusion induced transient focal cerebral ischemic rat model. Cerebral ischemia is a neurodegenerative disorder where insufficient blood flow to brain leads to cell death/excitotoxicity due to oxidative stress,Free radical production , inflammation. Ischemic condition led to alteration of multiple of targets and pathways including PI3K/AKT1, AKT1/GSK-3*β*/*β*-catenin, ERK1/2, apoptotic pathways, mTOR pathway (*Schölzke et al., 2007).* Amongst multiple targets, GSK-3*β* is one, which was found prominently active in ischemia. Up-regulation of GSK-3*β*, post translational phosphorylation, and down-stream signaling proteins were involved in propagating neuro degeneration during ischemia (*Bhat et al., 2012).*

Currently, clinical and pre-clinical studies of GSK-3 β inhibitors are in progress. Clinical trial (Phase-II) on lithium carbonate as a GSK-3 inhibitor is studied by Sao Paulo University and The National Institute of Neurological Disorder and Stroke for Alzheimer's disease (AD). A clinical trial for AD including GSK-3β inhibitors, NP12 and NP103, was filed by Noscira. A clinical trial on NP-12 (NP031112, tideglusib) has been completed for AD whereas; preclinical studies on GSK inhibitors CG-301338 as well as NP-103 are currently in progress (*Serenó et al., 2009*). In our study GSK3β competetive inhibitor AR-A 014418 was treated with two different doses 30µM/kg and 60µM/kg after 24hrs MCAo surgery. Further, In this study GSK3β non competetive inhibitor TDZD-8 was also used at a dose of 1500µM/kg. After occlusion of MCA following reperfusion injury MCAo group have exhibited increase in neurological deficit score (NDS) when compared to sham operated rats. The MCAo group have shown decreased locomtor activity in open field test, decreased exploratory, rearing , grooming, no of ambulations indicates that cerebral ischemia have good impact on locomotor activity and anxiogenic behaviour. Treatment with GSK3β inhibitor AR-A 014418 and TDZD-8 increased the locomotor activity.

In the elevated plus maze test evaluation of anxiety, MCAO animals showed reduced time spent and reduced number of entries in the open arms of maze in comparison to control rats. This increase in time and number of entries made in open arm has been correlated with anti-anxiety-like behaviour, reflecting a conflict between the rodent's preference for protected areas (closed arms of the maze) and their innate motivation to explore novel environments (open arms of the maze) (*Walf and Frye 2007).* In the present study, a positive correlation

between GSK3β inhibition and time spent in the open arm of the elevated plus maze explains reversal of the ischemia reperfusion injury-induced cognitive deficits and anxiety-like behaviour in the MCAO rats.

A study relating multiple pathways like calcium signaling (S100B – S100 calcium binding protein B) kinase pathway (MAPK/JNK-1, -2/p38), inflammatory pathway (NF-κβ, TNF-α, COX-2, iNOS and ICAM-1), and typical mitochondrial apoptosis pathway (SOD, cytochrome c caspase-9 and Bcl-2) were shown to have direct relation with GSK-3β in cerebral ischemia. Inhibition of GSK-3β showed neuroprotection through down-regulation of these multiple pathways *(Collino et al., 2008).* Recent studies have shown that GSK3 has role in inflammation by reducing translocation of CREB into the nucleus that leads to increases in the expression of pro-inflammatory cytokines such as In IL-1β and TNF-α *(Dylan et al.,2013*). In our study, treatment with GSK3β inhibitor AR-A 014418 and TDZD-8 downregulated Interleukin-1-Beta (IL-1β) and Tumor Necrosis Factor -1 alpha (TNF-α) level in brain suggest that the GSK3β inhibiton is beneficial in controling neuro inflammation in cerebral ischemic condition.

GSK-3β inhibition reduces infarct size in adult stroke models and further interacted with pro-apoptotic transcription factors, such as p53, which upregulates cytochrome C release and Bax levels providing more evidence of GSK-3β's pro-apoptotic role following brain injury (Chiu and Chuang, 2010). In this study, both competitive and non competitive inhibitors of GSK-3β reduced the pro apoptotic caspase 3 activity which is concordance with our previous study, GSK-3β inhibition inhibits caspase activity (*Darshi et al 2016).* GSK-3β activity is negatively regulated by several signal transduction cascades that protect neurons against apoptosis, including the phosphatidylinositol-3 kinase (PI-3 kinase) pathway. This suggests the interesting possibility that activation of GSK3β may contribute to neuronal apoptosis *(Friedrichs et al.,2011).* We found prominent down-regulation of AKT1/GSK-3 *β* pathway, where post-translational phosphorylation was reduced and made AKT1 inactive and GSK-3*β* (increased Ser 9 PGSK 3*β* levels)in active state. Being these molecules were GSK-3*β* inhibitor, it could not have effect on up-stream regulator AKT1. In contrast, both inhibitors showed post-translational phosphorylation in AKT1 and made in active state. These observations signified the regulatory loop between GSK-3*β* and AKT1 which played a role in activating AKT1 upon inhibition of GSK-3*β*. Activation of GSK-3*β* stabilizes *β*-arrestin 2,

PP2A complex, which is responsible for inactivation of AKT1 and vice versa (*O'Brien et al.,2011*).

7.Summary and conclusion

7. SUMMARY & CONCLUSION

- Neuroprotective activity of GSK-3ß inhibitors was evaluated in cerebral ischemia condition by exploring inflammatory and apoptotic pathways
- MCAo in rats was induced by occluding middle cerebral artery under anesthetic conditions and the behavioural parameters were studied at 24hrs and on 7^{th} day
- GSK- 3ß inhibitors of AR-A014418 (30 & 60 µm/kg) and TDZD-8 (1500 µm/kg) has been used as drug to test the neuroprotective action.
- The behavioural parameters include measurement of neurological deficit through grip strength measurement, anxiety behaviour in elevated plus maze and open field exploratory test.
- To support the neuroprotective mechanism of AR-A and TDZD-8 inflammatory markers IL-1ß, TNF-α were measured through ELISA and caspase activity assay, protein expression of GSK, PGSK, AKT, PAKT was done by Western blot method.
- Inducement of MCAo in rats have shown decreased locomotor activity, exploratory, rearing, grooming behaviour, indicates cerebral ischemia inducement in rats resulted in anxiogenic behaviour.
- The MCAo rats in elevated plus maze have shown increase in time spent in closed arm and decrease in time spent in open and centre area indicates increased anxiety.
- Post ischemic administration of AR-A significantly attenuated the behaviour characterised by increased locomotor activity, grip strength, grooming, rearing, no of entries and time spent in open arm suggesting the AR-A and TDZD-8 protects the brain damage induced by cerebral ischemia.
- Post ischemic administration of AR-A014418 (30&60µm/kg) and TDZD-8 (1500µm/kg) significantly reduced the IL-ß and TNF-alpha level. and significantly reduce the caspase-3 activity
- Post ischemic administration of AR-A014418(30&60µm/kg) increased the PAKT and PGSK levels
- Treatment with standard GSK-3β inhibitor AR-A014418 stabilizes AKT1/GSK-3β pathway, maintains GSK-3β in inactive state, prevents dephosphorylation thus prevents conversion of active caspase-3 or inhibits its activity.
- There is no difference between competitive, non-competitive inhibitors in the GSK-3 β mediated activity was observed.

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held at PSG IMS&R on 23-06-2016 as a participant

This activity has been reviewed and accepted by The Centre for Accreditation, The Tamil Nadu Dr. MGR Medical University and the University designates this educational activity for a maximum of 05 Credit points in Category II

Moderator

Dr. S. Ramalingam
Dean

KTN COLLEGE OF PHARMACY

(Affiliated to Kerala University of Health Science, Thrissur and Approved by AICTE & PCI, New Delhi)

Chalavara, Palakkad, Kerala-679505, Phone: 0466-2289500

Certificate

This is to certify that Prof/Dr/Mr/~~Mrs/Ms~~..... PANDIDURAI.T .....

P.S.G COLLEGE OF PHARMACY, COIMBATORE .....*has participated as Resource person/Delegate/Member of organizing Committee, Chairperson in the National Seminar on "CURRENT SCENARIO IN DRUG DISCOVERY & DEVELOPMENT" (CSDDD) organized by Department of Pharmacology, KTN College of Pharmacy & Funded by the Science and Engineering Research Board (SERB), DST, Govt. of India in 3rd & 4th March 2017*

Mr. Ramkumar. R.P
Scientific Co-ordinator

Dr. L. Sutharson
Convener & Organizing Secretary

Mr. P.P. Premkrishnan
Patron

PSG Institute of Medical Sciences & Research
Institutional Animal Ethics Committee

Registration No. : 158 / PO / ReBi / SL / 99 / CPCSEA
POST BOX No. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2570170, 2598822 Fax : 91 422 - 2594400 Email : psganimalethics@gmail.com

DATE: 21.11.2016

Title of the Project: Inflammatory pathway studies in neurodegenerative condition with reference to cerebral ischemia. .

Proposal Number: 345 /2016/ IAEC

Name of the Applicant: Dr.M.Ramanathan

Approval date: 21.11.2016

Expiry date (Termination of the Project): 20.11.2017

Methodology: Approved.

Name of species: **Swiss albino mice/ Wistar rats/ Sprague Dawley rats/ Guinea pigs/ Newzealand White rabbits.**

Male/Female/Both sex----------SD rats 63 male----------animals approved.

Signature of Chairperson

Date: 21.11.16

Dr.M.Ramanathan

Name of the chairperson

The Chair Person, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

Signature of the CPCSEA nominee

Date: 21/11/2016.

Dr.C.Kathirvelan

Name of IAEC/CPCSEA nominee

Main Nominee, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.